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14. ABSTRACT

My experiments indicate that Serum and Glucocorticoid regulated Kinase (SGK) proteins facilitate breast cancer invasive migration, a critical step for ultimate cancer metastasis to other organs. The activation of SGK during cellular stress conditions, such as low oxygen found within a tumor, makes this data increasingly imperative for therapeutics. The research shown here demonstrates SGK loss in highly mestatic breast cancer cell lines causes an invasive migration defect. Conversely, the overexpression of SGK isoforms in breast cancer cell lines causes an enhancement of invasive migration. These discoveries are helping to elucidate new mechanisms that can be targete for more specific and successful therapies to block breast cancer metastasis. Known targets of SGK proteins are being examined for their contribution to the metastatic properties of breast cancer cells. These studies will determi the importance of SGK proteins as putative therapeutic targets for breast cancer motility.

15. SUBJECT TERMS
Serum and Glucocorticoid Regulated Kinase (SGK), breast cancer cell migration and invasion, retroviral overexpression, lentiviral silencing

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Introduction

Breast cancer arises as a result of alterations in the cell signaling networks that control growth, division, and metastasis. Knowledge of the details of these signaling systems is becoming increasingly essential for the design of new and effective treatments. One such pathway is phosphoinositide 3-kinase (PI3K) which activates numerous downstream effectors, which in turn regulate a plethora of physiological and pathophysiological process. In human cancer, two critical PI3K effectors are the protein kinases Akt, the human homologue of the viral oncogene *v-Akt,* and SGK, the Serum and Glucocorticoid-regulated Kinase. The importance of this pathway in disease is underscored by the fact that the gene encoding the catalytic subunit of PI3K, *PIK3CA*, is one of the most frequently mutated oncogenes in breast cancer. The two most common *PIK3CA* somatic oncogenic mutations occurring in breast cancer are E542K, in the helical domain, and H1047R, in the kinase domain, and both mutations result in hyperactive PI3K signaling (1). The oncogenic *PIK3CA* mutations have been documented at a frequency 25-40% in breast tumors (2,3). Therefore, evaluating the function of downstream effectors of PI3K in breast cancer, such as Akt, has been a cancer biology focus for some time. Numerous studies have revealed PI3K and Akt as a central signaling node integrating processes of cell growth, survival, and proliferation. Recently our laboratory discovered that distinct isoforms of Akt (Akt1 and 2) also play a key role in breast cancer cell invasive migration, whereby Akt1 inhibits and Akt2 enhances invasive migration (4). Despite the wealth of knowledge concerning PI3K and Akt signaling, relatively little is known about SGK, also a PI3K effector. SGK isoforms share many characteristics with Akt, such as high homology in the catalytic domain, common mechanism of phosphorylation and activation, a significant number of overlapping substrates, and deregulation in breast carcinoma (5,6). To date, no studies have investigated any role for SGK in cell invasive migration or metastasis. This is despite the fact that SGK is also activated by oncogenic PI3K and that SGK isoforms are documented as amplified with high frequency in breast cancer(6). This makes the similarities and distinctions between Akt and SGK attractive targets for therapeutic applications in cancer.

Body

The following tasks from the Statement of Work for this project were the focus for the research period from March 15, 2012- March 14, 2013:

Task 1: Examine the influence of SGK isoforms on cellular motility both individually and in concert with Akt isoforms (months 1-27)

1.4 Overexpress SGK and Akt isoforms individually in normally migratory breast cancer cell lines and normally static breast cell lines and evaluate motility changes via Transwell migration and Transwell Matrigel invasion assays (months 8-10)

1.5 Overexpress SGK isoforms in concert with Akt isoforms and evaluate motility changes with Transwell migration and Transwell Matrigel invasion assays in normally migratory breast cancer cell lines and normally static breast cell lines (months 11-13)

1.6 Silence SGK expression, individually and in concert with Akt, using lentiviral siRNA constructs and evaluate motility changes with Transwell migration and Transwell Matrigel invasion assays in normally migratory breast cancer cell lines and normally static breast cell lines (months 14-17)

1.7 Run Transwell migration assays and Transwell Matrigel invasion assays using PIK3CA mutant MCF10A cells and optimized SGK1/3 and Akt1 and Akt2 siRNA (months 18-21)

1.8 Make SGK known substrate phosphorylation mutants and optimize their expression (month 22-23)

1.9 Run Transwell migration assays and Transwell Matrigel invasion assays with SGK substrates mutants in background of SGK overexpression and silencing to examine SGK substrate invasive migration importance to SGK invasive migration phenotype (months 24- 27)

Task 2: Identify unique Akt and SGK substrates in an isoform-specific manner (months 28-36)

2.1 Prepare SGK1, SGK3, and SGK1/3 silenced MDA-MB231 for murine injections (month 28)

Progress

1.2 Optimize lentiviral infection technique and siRNA silencing sequences for Akt and SGK isoforms in breast carcinoma cell lines (months 2-3)

Silencing of SGK and Akt isoforms had been confirmed March 15, 2010- March 14, 2011. However on further analysis of substrate NDRG1 it appears the silencing of SGK3 but not Akt isoforms causes an increase in total NDRG1 protein level while simultaneously decreasing phosphorylation of NDRG1 at T346/356/366 SGK phosphorylation sites (further discussed in aim 1.8) (FIGURE 1).

1.3 Optimize lentiviral infection for PIK3CA and refractory PIK3CA overexpression in MCF10A breast cells (month 4-7)

March 15, 2010- March 14, 2011 I had identified 2 PIK3CA/pLKO shRNA constructs that efficiently silenced PIK3CA expression (FIGURE 2) and transfection overexpression system that worked efficiently in 293T cells. At the start of March 15, 2011 we tested the same overexpression vectors of PIK3CA and its two most common breast cancer mutation hotspots, E545K and H1047R in MCF10A breast cells but expression was insufficient to get measurable signal of SGK3 activity by western blot We then regressed to use 293T cells for overexpression and in vitro kinase assays to confirm the effect of p110a activity upon SGK3 kinase activity. (FIGURE 3). Upon transfection of E545K and H1047R PIK3CA mutations in 293T cells a significant increase the activity of exogenous SGK3 was observed measured by SGK3 pulldown and *in vitro* kinase assay upon known substrate GST-GSK3beta and its phosphorylation at Serine 9 known SGK3 site. However, positive control pAkt S473 which is known to function significantly downstream of PIK3CA, did not show changes in phosphorylation. For this reason we have moved to a retroviral overexpression system which will allow more consistent PIK3CA overexpression and allow us to infect MCF10A breast cells which did not transfect well. Use of the new system will allow us to examine the overexpression of PIK3CA mutants on SGK3 activity in a more relevant breast cell system. PIK3CA mutants have been made in the JP1520 retroviral system.

1.6 Silence SGK expression, individually and in concert with Akt, using lentiviral siRNA constructs and evaluate motility changes with Transwell migration and Transwell Matrigel invasion assays in normally migratory breast cancer cell lines and normally static breast cell lines (months 14-17)

SGK3 shRNA and Akt 2 shRNA both dramatically inhibit MCF7 breast cancer cell migration although SGK3 shRNA inhibits migration to a greater extent than Akt2 shRNA. The difference in SGK3 and Akt2shRNA inhibition is statistically significant. Upon dual knockdown of Akt2 and SGK3 upon co-infection with shRNAs and selection the inhibition is equivalent to SGK3 knockdown alone and does not inhibit to a greater extent (FIGURE 4). This could be explained by redundant substrates or that the level of migration was already inhibited to its maximum by SGK3 alone. To further examine the redundancy of the two kinases both SGK3 and Akt2 will be silenced in a cell line that exhibits higher degrees of migration such as T47D breast cancer cells, which will thus enable us to identify a greater number of differences in invasive migration inhibition and substrate regulation.

1.7 Run Transwell migration assays and Transwell Matrigel invasion assays using PIK3CA mutant MCF10A cells and optimized SGK1/3 and Akt1 and Akti siRNA (months 18-21)

Transwell migrations and invasions have been run with the new retrovirally infected overexpression MCF10A PIK3CA mutant cells (See Aim 1.3). These cells and experiments are in the process of being evaluated and repeated.

1.8 Make SGK known substrate phosphorylation mutants and optimize their expression (month 22-23)

NDRG1 has been shown to be a potent mediator of invasive migration through its roll in the recycling of E-cadherin the cellular membrane(7). However, the roll of SGK3 phosphorylation in the regulation of NDRG1 activity was not known. We mutated the 5 SGK phosphorylation sites in NDRG1-FLAG in various combinations to understand their role and their seeming redundance in closely adjacent sites within the NDRG1 C-terminus. We made NDRG1 2A- T328A/T330A, NDRG1 3A- T346A/T356A/T366A and NDRG1 5A-T328A/T330A/T346A/T356A/T366A and were able to see reproducible overexpression with transient transfection 293T cells. Upon SGK3 silencing we were able to see an increase in total NDRG1 with a simultaneous decrease in phosphorylation (FIGURE 1). We therefore examined the stability of NDRG1 with cyclohexamide treatment and were able to see a decrease in NDRG1 levels with the inhibition of protein synthesis in MCF10A breast cancer cells (FIGURE 5). We then treated MCF10A cells with MG132 and were able to see a significant increase in total NDRG1 with 18 hour inhibition of 26S proteasome degradation activity. NDRG1, is a known metastasis suppressor so SGK phosphorylation leading to its degradation is a novel mechanism in which SGK3 regulates invasive migration, which Akt does not share.

1.9 Run Transwell migration assays and Transwell Matrigel invasion assays with SGK substrates mutants in background of SGK overexpression and silencing to examine SGK substrate invasive migration importance to SGK invasive migration phenotype (months 24- 27)

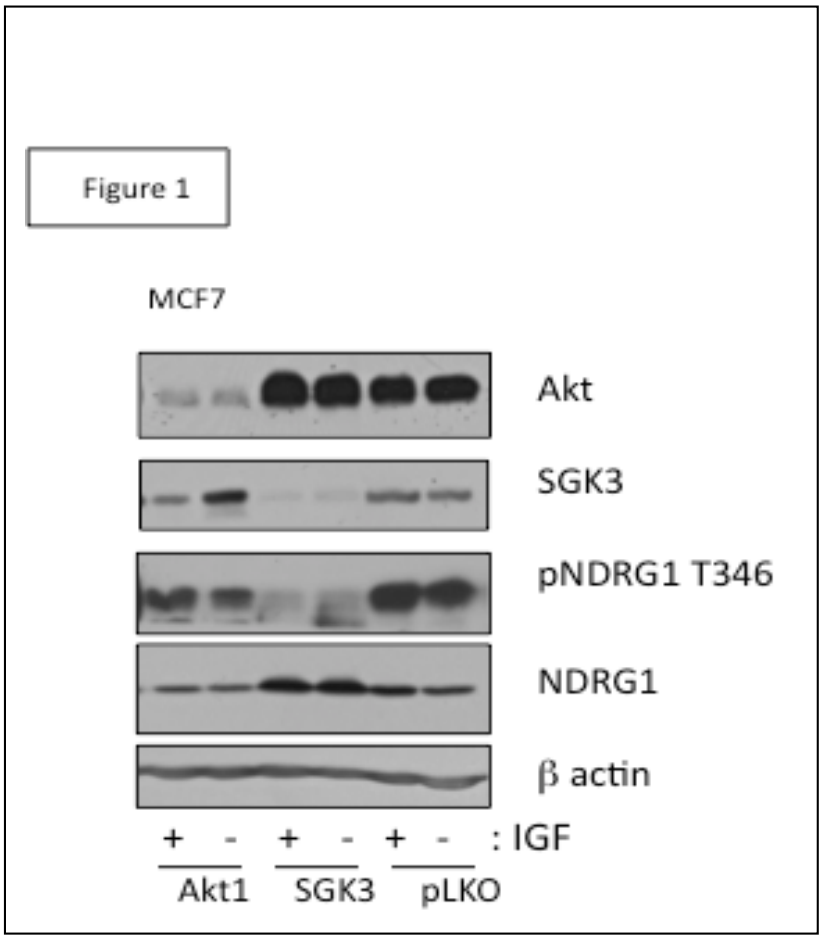
NDRG1 wildtype was initially ran in a migration assay in MCF7 breast cancer cells in order to confirm previously data implicating NDRG1 as an inhibitor of migration. NDRG1 significantly inhibited MCF7 migration. With the addition of SGK inhibitor GSK650394 MCF7 cells were inhibited to a greater extent thus implying SGK3 may be enhancing migration through its enhancement of NDRG1 degradation (FIGURE 6).

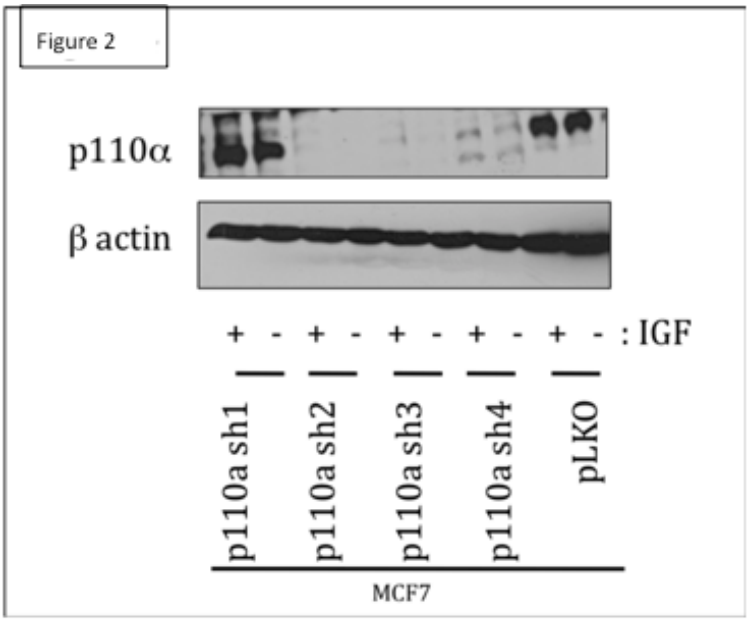
Task 2: Identify unique Akt and SGK substrates in an isoform-specific manner (months 28-36)

2.1 Prepare SGK1, SGK3, and SGK1/3 silenced MDA-MB231 for murine injections (month 28)

SGK3 expression in MDA-MB231 cells was very low on initial examination (FIGURE 7 and 8). After validation of SGK3 phospho-T320 antibody, SGK3 phosphorylation and thus activity was also low. SGK3 does not appear to active nor respond to phosphorylation by IGF or the addition of

PIK3CA mutant overexpression. Alternate breast cancer cell lines were thus deemed an appropriate alternate approach before proceeding into the more extensive animal model system. MCF7 breast cancer cells were chosen due to their high expression of SGK3 and ease of manipulation by lentivirus and retrovirus systems.





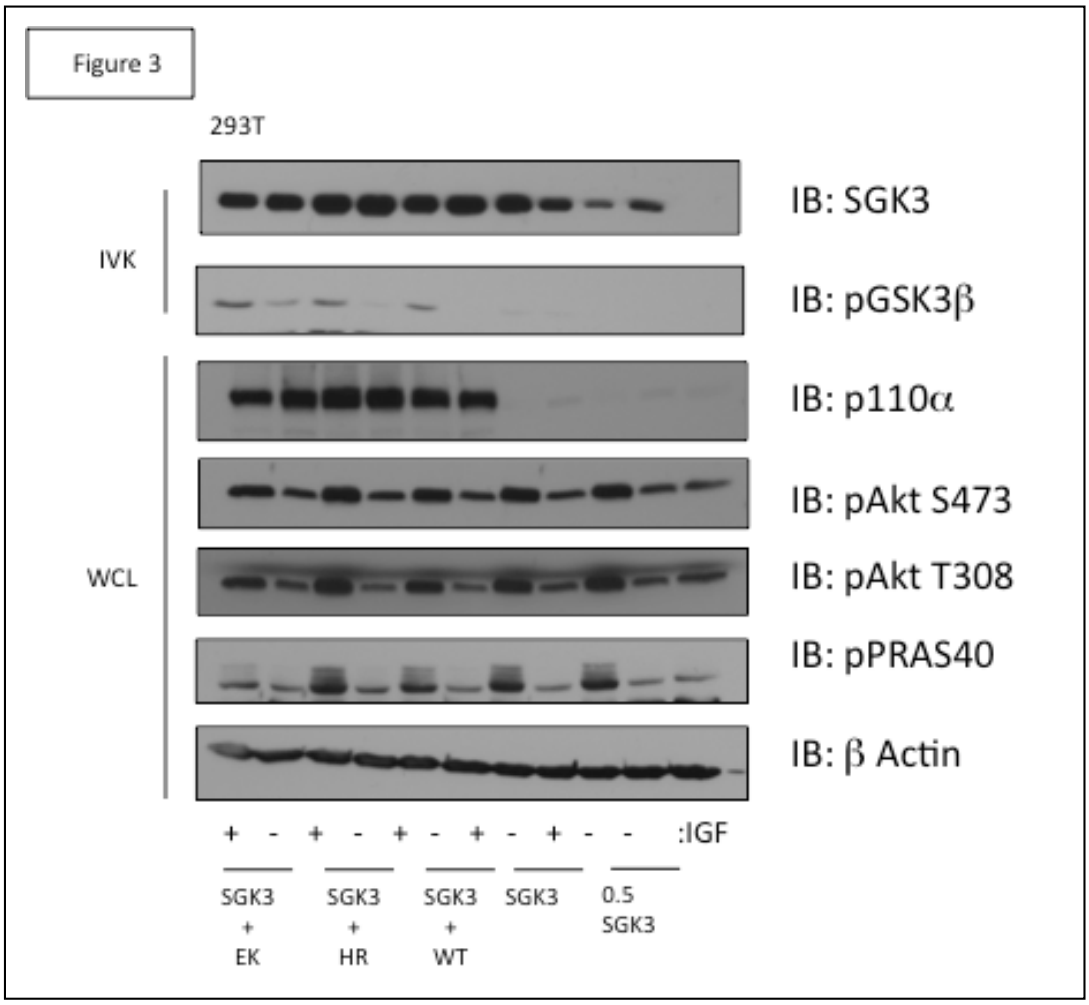


Figure 3
293T
IVK
WCL
IB: SGK3
IB: pGSK3β
IB: p110α
IB: pAkt S473
IB: pAkt T308
IB: pPRAS40
IB: β Actin
+ - + - + - + - + - - :IGF
SGK3 + EK
SGK3 + HR
SGK3 + WT
SGK3
0.5 SGK3

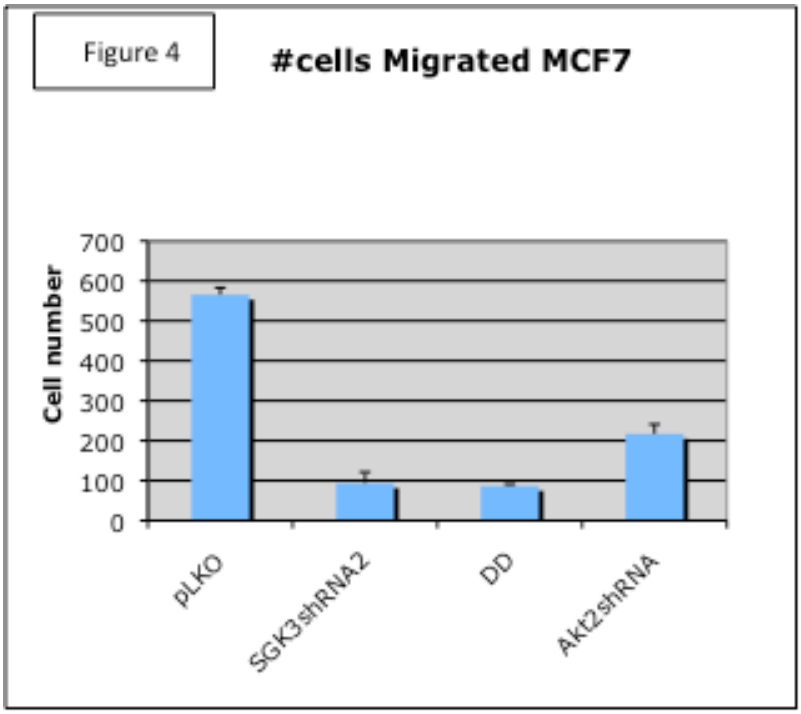


Figure 4
#cells Migrated MCF7
Cell number
700
600
500
400
300
200
100
0
pLKO
SGK3shRNA2
DD
Akt2shRNA

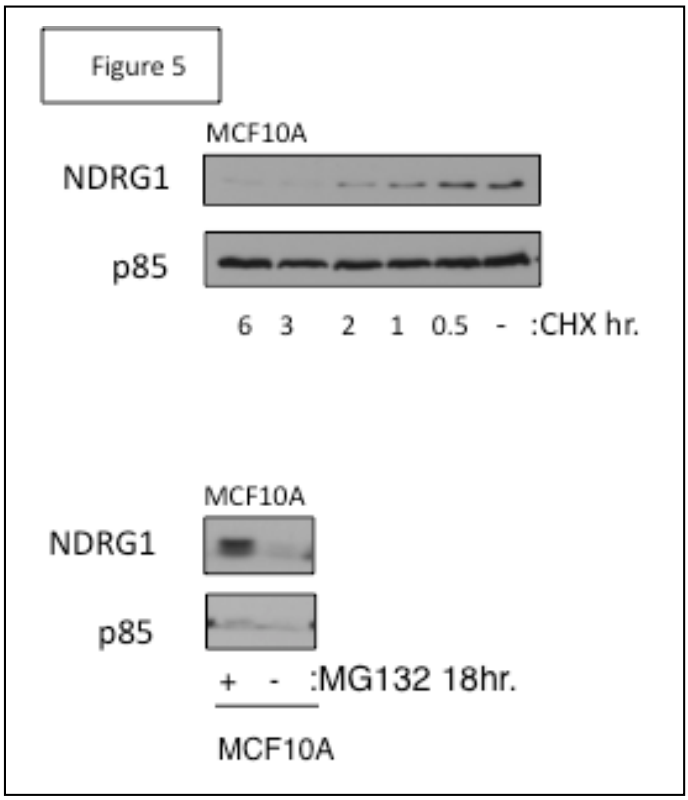


Figure 5
MCF10A
NDRG1
p85
6 3 2 1 0.5 - :CHX hr.
MCF10A
NDRG1
p85
+ - :MG132 18hr.
MCF10A

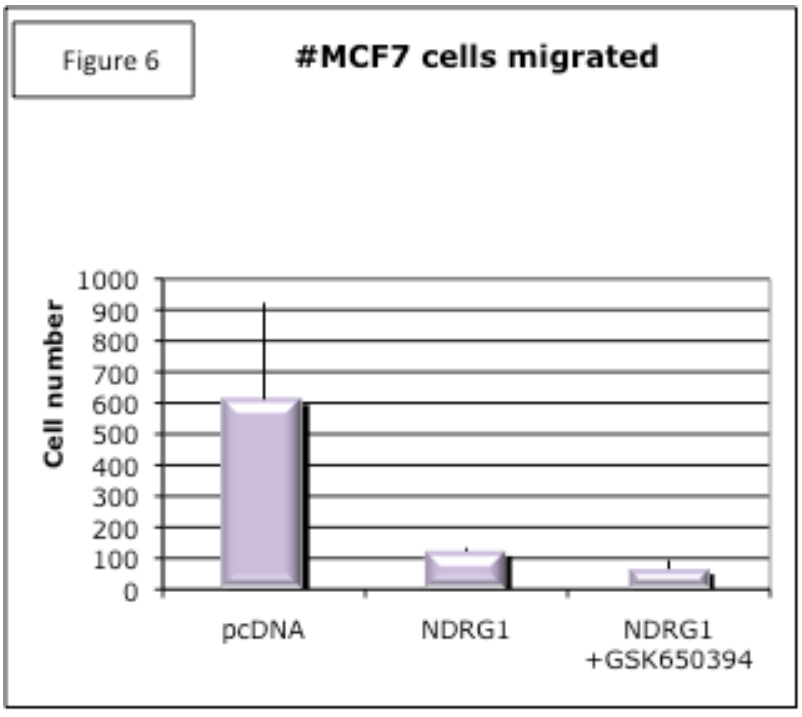


Figure 6
#MCF7 cells migrated
Cell number
1000
900
800
700
600
500
400
300
200
100
0
pcDNA
NDRG1
NDRG1
+GSK650394

Figure 7

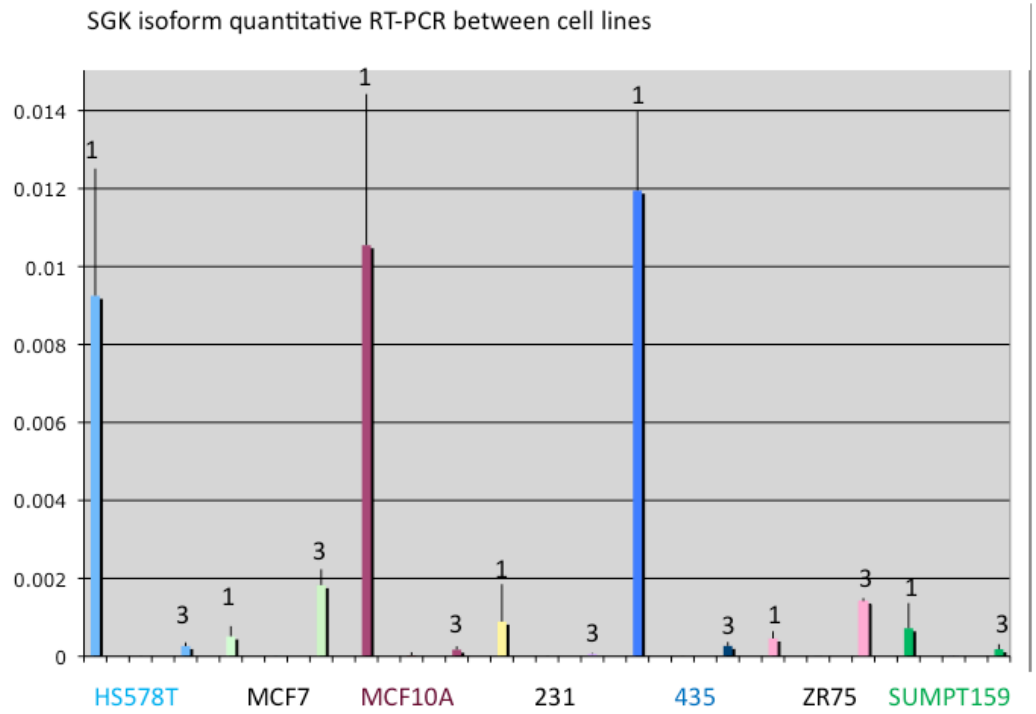
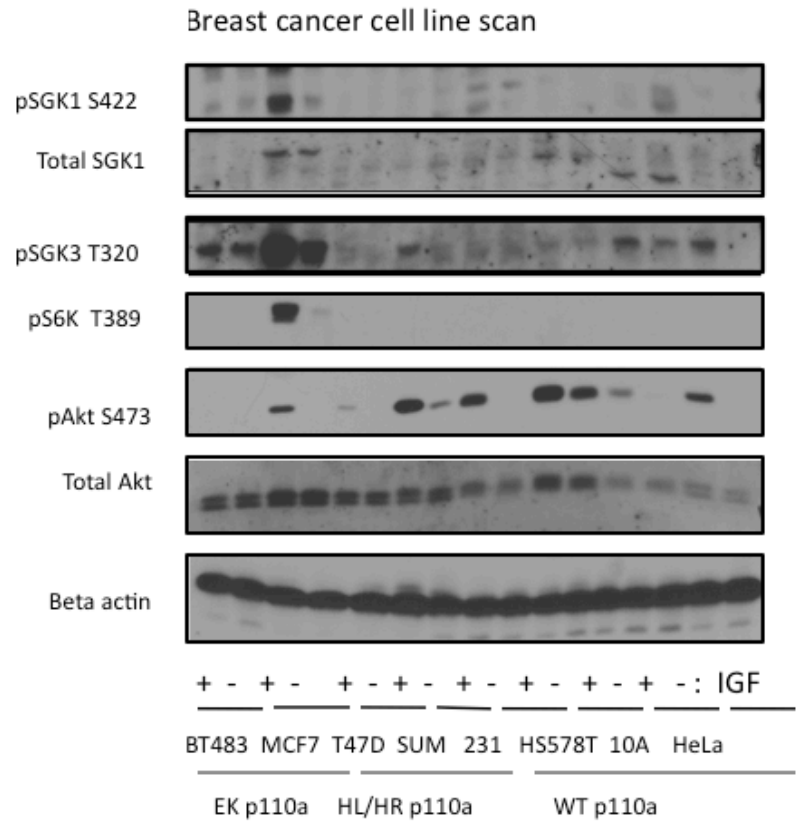


Figure 8



Current research and future directions

Current experimental efforts were directed on establishing the contribution and importance of NDRG1 in regulating the SGK isoform invasive migration phenotypes in breast cancer cell lines, specifically in cancer cells known to be deficient in Akt regulation of the PIK3CA pathway, such as MCF7 breast cancer cells which contain a PIK3CA E545K mutation, which is the commonly mutated gene in breast cancer. Understanding the ability of NDRG1 to mediate SGK3 phenotypic rolls in the invasive migration of breast cancer cells will be another prominate focus prior to commencement of work in murine models.

Key Research Accomplishments

- **Reproducible migratory enhancement by SGK3 in breast cancer cells was discovered**
- **Concordant enhancement of invasion by SGK3 in Matrigel Transwell invasion assays was established**
- **Effective lentiviral knockdown system created with 2 efficient shRNAs towards each SGK1 and SGK3 with corresponding refractory SGK1 and SGK3 overexpression constructs in order to do future rescue experiments to confirm SGK significance in invasive migration of breast cancer.**
- **Efficient PIK3CA overexpression and lentiviral knockdown constructs were made and validated.**
- **Overexpression of PIK3CA breast cancer hot spot mutations, H1047R and E545K, were shown to significantly enhance SGK3 phosphorylation in addition efficient overexpression and knockdown systems were established for PIK3CA**
- **NDRG1 was shown to be a substrate contributing to SGK isoform invasive migration phenotype independent of Akt and Akt substrates.**

Reportable Outcomes

N/A

Conclusion

SGK1 and SGK3 have been shown in a number of breast cancer cell lines to enhance both migration and invasion using Transwell migrationa and Transwell Matrigel invasion assays. SGK1 and SGK3 shRNAs were subsequently made and validated and will be used to determine the importance of normally metastatic breast cancer cell utilization of SGKs. Additionally PIK3CA overexpression and knockdown constructs were made and validated. The breast cancer hot spot mutations in PIK3CA

commonly mutated in breast cancer tumors were shown upon overexpression to activate SGK3 while wild type overexpression caused no change in SGK3 activity. The placement SGK1 and SGK3 downstream of common breast cancer mutations with now demonstrated metastatic characteristics make SGK1 and SGK3 promising future molecular targets for breast cancer therapeutics. We further identify SGK3 as a putative mediator of invasive migration through its phosphorylation of NDRG1 leading to its subsequent degradation. NDRG1 thus functions as a suppressor of invasive migration through known pathways such as its role in the recycling of E-cadherin the plasma membrane.

Appendices

N/A

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